REMARKS/ARGUMENTS

Claims 1, 3, 4, 10-19, 37, 38 and 40-51 are active in this application. Support for

Claims 40-51 is found in original Claims 5-7 and the specification on page 6, lines 17-20. No

new matter is added by these amendments.

Applicants thank the Examiner for allowing Claims 3, 4, 12, 14, 16, 18 and 37.

Applicants also thank the Examiner for discussing proposed amendments with the

Applicants' undersigned representative to address the rejections raised in the Office Action.

Based on this discussion, it is believed that the amendment submitted herein to Claims 1 and

10 address the issues in the Office Action. Accordingly, withdrawal of the objection of

Claims 1 and 5, the rejection of Claims 10-11 under 35 U.S.C. § 112, second paragraph, and

the objection to Claims 13, 15, 17 and 19 and 38 under 37 C.F.R. § 1.75 is requested.

Since a rejection was made previously under 35 U.S.C. § 112, first paragraph both

based on written description and scope of enablement of claims similar to Claims 40-51,

Applicants note the following.

First, the claims are drafted in accordance with the Examiner's suggestion on page 5

of the Office Action mailed July 21, 2003. In addition, the isolated polynucleotide in Claims

40-51 encode a protein having LuxR transcriptional activation activity.

With respect to the scope of enablement of these claims, it would not require undue

experimentation to make and/or use polynucleotides are 70% identical and encode a protein

having the activity required in the present claims. The specification describes SEQ ID NO:1,

LuxR transcriptional activation activity, and demonstrates its role in amino acid production.

During the above-noted discussion, the Examiner indicated that further information

describing domains or the active regions of the luxR protein would be helpful in assessing the

issues under 35 U.S.C. § 112, first paragraph.

Accordingly, Applicants attach hereto a publication demonstrating such knowledge--

Fuqua et al. (1994), *J. Bacteriology*, 176(2):269-275. This publication reviews the LuxR

family of transcriptional regulators and particularly point out the regions required fro DNA

binding and activation. See, for example, Figure 2 on page 270; Figure 3 on page 272; and

the discussion on page 273, first column, third and fourth paragraphs.

Therefore, claims 40-51 are described and enabled based on the specification as

originally filed coupled with the knowledge available in the art.

Accordingly, Applicants request an indication that all pending claims are allowable.

Early notice of such is also requested.

Respectfully submitted,

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7

# MINIREVIEW

## Quorum Sensing in Bacteria: the LuxR-LuxI Family of Cell Density-Responsive Transcriptional Regulators†

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It has long been appreciated that certain groups of bacteria exhibit cooperative behavioral patterns. For example, feeding and sporulation of both myxobacteria and actinomycetes seem optimized for large populations of cells behaving almost as a single multicellular organism. The swarming motility of microorganisms such as *Vibrio parahaemolyticus* and *Proteus mirabilis* provides another excellent example of multicellular behavior among bacteria (2). Intercellular communication likewise has been appreciated for several years in *Vibrio fischeri*, *Myxococcus xanthus*, *Bacillus subtilis*, *Streptomyces* spp., the eukaryotic slime mold *Dictyostelium discoideum*, and other species (44). Here we first review how the marine luminescent bacterium *V. fischeri* uses the LuxR and LuxI proteins for intercellular communication and then describe a newly discovered family of LuxR and LuxI homologs in diverse bacterial species.

## AUTOINDUCTION OF BACTERIAL LUMINESCENCE

Autoinduction of luminescence in the marine bacteria *V. fischeri* and *Vibrio harveyi* was described in the early 1970s (26, 57). When these bacteria are cultured in broth, they exhibit a lag in luminescence gene (*lux*) expression during early and mid-exponential growth, followed by a rapid increase in expression during the late exponential and early stationary growth phases. Luminescence in early-log-phase cultures is induced by the addition of cell-free fluid extracts from stationary-phase cultures. Furthermore, the extracts show strain specificity, in that the addition of a *V. harveyi* extract to an early-log-phase culture of *V. fischeri* (or vice versa) does not induce luminescence (26, 55, 57).

Autoinduction should not be confused with autoregulation or autorepression, two similar terms that describe entirely different phenomena (for a review of autoregulation, see reference 49). Autoinduction defines an environmental sensing system that allows bacteria to monitor their own population density. The bacteria produce a diffusible compound termed autoinducer which accumulates in the surrounding environment during growth. At low cell densities this substance is in low concentration, while at high cell densities this substance accumulates to the critical concentration required for activation of luminescence genes (26, 55).

The *V. fischeri* autoinducer (VAI) is 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)hexanamide (27), which is commonly referred to as *N*-3-(oxohexanoyl)homoserine lactone (Fig. 1). The cell membrane is permeable to VAI, and thus, it accumulates in the growth medium (45). At low cell densities, VAI passively diffuses out of cells down a concentration gradient, while at high cell densities, VAI accumulates (at an intracellular concentration equivalent to the extracellular concentration). A concentration on the order of 10 nM is sufficient to activate transcription of the luminescence genes (45). *V. fischeri* is the specific symbiont in the light organs of certain marine fishes and squids (for recent reviews, see references 24, 51, and 68) and also occurs free-living in sea water. In light organs *V. fischeri* achieves high cell densities ($10^{10}$ to $10^{11}$ cells per ml) and is luminescent, while in sea water this bacterium is found at a density of less than $10^2$ cells per ml and should not be luminescent (8, 24, 56, 67–70). Therefore, the autoinduction system allows *V. fischeri* to discriminate between the free-living (low cell density) state and host-associated (high cell density) state and to induce the luminescence system only when host associated.

Cloning a fragment of *V. fischeri* DNA that encodes the functions required for autoinducible luminescence in *Escherichia coli* was first reported by Engebrecht et al. (29a). The luminescence genes are organized in two divergently transcribed units whose start sites are about 150 bp apart. One unit contains *luxR*, which encodes the 250-amino-acid LuxR protein, the transcriptional activator of luminescence. The other unit is an operon, *luxICDABEG*, and is activated by LuxR in the presence of VAI. A sequence having a dyad symmetry is found centered at about −40 bp upstream from the start of *luxICDABEG* transcription, and mutational analysis of this sequence demonstrates that it is required for *luxICDABEG* activation by LuxR (18). This putative LuxR-binding site or *lux* box is also required for rather subtle (two- to threefold) *luxR* autoregulation which can be either positive or negative, depending on the cellular levels of VAI and LuxR (23, 73).

The *luxI* gene encodes a 193-amino-acid protein that directs *E. coli* to synthesize VAI and is therefore considered to be the autoinducer synthase (29a, 30). The other genes in the *luxIC-DABEG* operon play mechanistic roles in light production. *luxA* and *luxB* code for the α and β subunits of luciferase. *luxC*, *luxD*, and *luxE* code for components of the fatty acid reductase complex required for synthesis of the aldehyde substrate for luciferase (30). *luxG* is not required for luminescence in *E. coli*, but it is thought that it might code for a flavin mononucleotide reductase that generates reduced flavin mononucleotide as a substrate for luciferase (3).

*luxR* and *luxI* mediate cell density-dependent control of *lux* gene transcription. At low cell densities, *luxI* is transcribed at a

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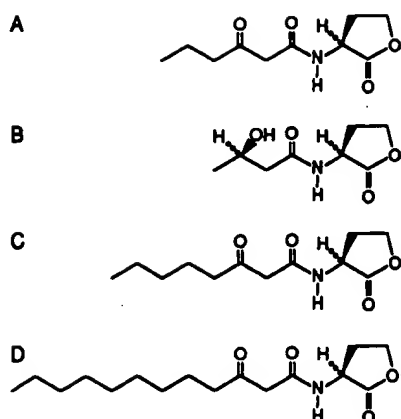
A

B

C

D

FIG. 1. Structures of VAI and related signals produced by other bacteria. (A) VAI, N-(3-oxohexanoyl)-L-homoserine lactone; (B) HAI, the V. harveyi autoinducer, N-(3-hydroxybutanoyl)-L-homoserine lactone; (C) AAI, the A. tumefaciens autoinducer, N-(3-oxooctanoyl)-L-homoserine lactone; (D) PAI, the P. aeruginosa autoinducer, N-(3-oxododecanoyl)-L-homoserine lactone.
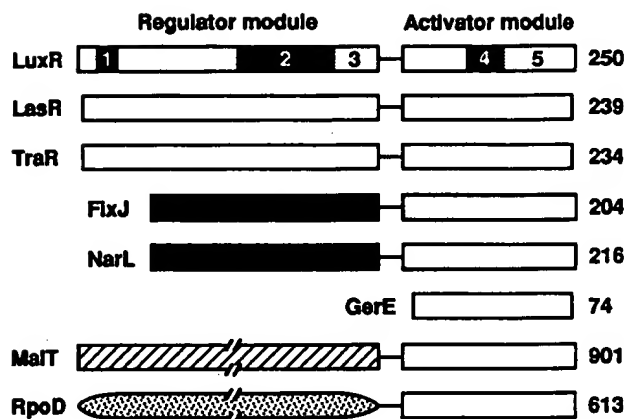


FIG. 2. Schematic diagram of LuxR and representative members of the LuxR superfamily of transcription factors. The top shows the regulator and activator modules of LuxR. The numbers within each module of LuxR indicate specific regions as follows: 1, the luxR autoregulation region (this small region is required for control of luxR by the LuxR protein, but it is not required for DNA binding or autoinduction of luxICDABE); 2, autoinducer-binding region; 3, multimerization region; 4, HTH, DNA-binding region; 5, C-terminal region required for transcriptional activation (11–13, 75, 77). LasR and TraR are described in the text. The remaining proteins show sequence similarity with LuxR in the C-terminal module only. These proteins include FixJ from R. meliloti (38) and NarL from E. coli (78), which are response regulators in the two-component family of regulators (59); GerE from B. subtilis (38), which consists wholly of the C-terminal module; the maltose catabolism regulator MalT from E. coli (38); and region 4 (the −35 binding domain) of $\sigma^{70}$ and other $\sigma$ factors (43). Sizes in amino acid residues are indicated to the right of each protein.

basal level and VAI accumulates slowly in the growth medium. At a sufficiently high VAI concentration, this signal compound interacts with LuxR, which then activates transcription of the luxICDABEG operon. This results in the induction of luminescence and in the positive autoregulation of luxI. The significance of luxI autoregulation is not known, and it has not been demonstrated experimentally that luxI induction leads to an increase in the rate of VAI synthesis. In addition to LuxR and LuxI, a number of other factors influence lux gene expression in V. fischeri. Recent reviews on these regulatory elements have been published elsewhere (24, 52, 53), and these regulatory elements will not be covered here.

**LuxR, the cell density-dependent transcriptional activator.** To date, there are no reports describing the activity of LuxR or any of its homologs in vitro. Nevertheless, molecular genetic analyses of LuxR, most of which have been performed in E. coli, have allowed the development of a general view of the mechanism of autoinduction and of the structure and function of LuxR. The LuxR polypeptide requires GroEL and GroES for folding into an active conformation (1, 19). LuxR appears to be a two-domain polypeptide (Fig. 2).

A series of LuxR proteins containing single-amino-acid alterations or deletions in the amino or carboxyl terminus have allowed mapping of specific functions to discrete regions of the protein (11–13, 75, 77). Amino acids at the extreme amino terminus (within residues 10 to 20) are required for repression of the luxR gene, but not for binding to the lux box or activation of luxICDABEG. Amino acids in the region of 20 to 156 define a regulator module, since removing residues 2 to 156 results in high-level, autoinducer-independent luxICDABEG transcription. This result indicates that the C-terminal region is sufficient for DNA binding and transcriptional activation and strongly suggests that the amino-terminal half of the protein plays some inhibitory role that is neutralized by autoinducer. Single-amino-acid alterations between residues 79 and 127 appear to alter putative interactions with VAI. Dominance studies suggest that binding of VAI to LuxR stimulates multimer formation and binding to lux boxes (11, 13). Residues in the region of 116 to 161 appear to be critical for multimer formation (13). The sequence from residues 190 to 210 contains a helix-turn-helix (HTH) motif with similarity to the

DNA-binding regions of several other transcription factors (38) (Fig. 2). Residues in the carboxyl terminus (the so-called carboxy-terminal tail, residues 230 to 250) are required for activation of luxICDABEG but not required for autoregulation of luxR. This result implies that the residues 230 to 250 are not required for DNA binding but may be needed to make contacts with RNA polymerase necessary for activation (12).

LuxR does not possess any characteristic membrane-spanning sequences, yet recent results indicate that LuxR is localized to the cytoplasmic membrane (47). Thus, it joins a growing list of such transcriptional activators (16, 37, 50, 54, 82). Although little is known about the LuxR-membrane association, it is hypothesized that the N-terminal module contacts the membrane and the C-terminal transcriptional activator module extends into the cytoplasm. One speculation is that binding of VAI to LuxR is facilitated by protein-lipid contacts. Similar proposals have been made to describe how lipophilic flavonoids interact with the membrane-localized NodD protein of Rhizobium meliloti (65, 72).

There has been one study on the influence of VAI analogs on LuxR control of luminescence gene transcription in V. fischeri (29). VAI analogs with N-acyl side groups that differ from the natural six-carbon N-acyl group and VAI analogs with substitutions in the carbonyl groups show reduced activity. Molecules with substitutions for the oxygen atom in the homoserine lactone ring abolish autoinduction. Many non-stimulatory autoinducer analogs can inhibit the activity of VAI, suggesting that they bind LuxR and prevent VAI binding. It is possible that VAI binding to LuxR involves an interaction of the N-acyl group with a hydrophobic pocket in LuxR. N-acyl groups of the proper length may position the homoserine

lactone ring such that interactions of the ring with specific amino acid residues could occur.

**LuxI, the cell density signal generator.** The *luxI* gene directs both *V. fischeri* and *E. coli* to synthesize VAI (25, 31), suggesting strongly that it encodes VAI synthase. The LuxI protein has not been studied in vitro, but cellular extracts of *V. fischeri* have been shown to catalyze the synthesis of VAI from *S*-adenosylmethionine and 3-oxohexanoyl coenzyme A (28). The concentrations of 3-oxohexanoyl coenzyme A required to saturate the enzyme activity are high, suggesting that 3-oxohexanoyl acyl carrier protein might be the true substrate for VAI synthase (28). Additionally, synthesis of the *V. harveyi* autoinducer, which we term HAI (see below), was shown to utilize specifically the D isomer of its fatty acid precursor, suggesting that the acyl moieties of autoinducer are derived directly from lipid biosynthetic intermediates (10). Studies of the biosynthesis of VAI and of the structure and function of LuxI should provide fertile ground for future investigations.

**Bioluminescence in *V. harveyi*.** The structure of HAI was published in 1989 (9). Although the structure of HAI is strikingly similar to that of VAI (Fig. 1), VAI and HAI do not show cross-functionality (26, 36, 57). The structural relationship between VAI and HAI suggested that *V. harveyi* should possess genes homologous to the *V. fischeri luxI* and *luxR* genes, which encode a signal generator and an AI-responsive transcriptional activator of bioluminescence genes, respectively. Unfortunately the genes responsible for cell density control of *V. harveyi* luminescence are not linked to its *lux* operon, and these genes have eluded identification. A gene encoding a transcriptional activator controlling luminescence in *V. harveyi* has been described and termed the *V. harveyi luxR* gene. However, the *V. harveyi luxR* gene product does not respond to HAI nor does it control luminescence in a cell density-dependent manner (76, 80). Understanding the molecular basis of autoinduction in *V. harveyi* awaits further investigation.

## HOMOLOGOUS REGULATORY SYSTEMS

Within the past 2 years it has become clear that several bacterial genera contain regulatory systems homologous to the LuxR and LuxI proteins. In two systems, both a signal generator homologous to LuxI and a transcriptional activator homologous to LuxR have been described. These systems regulate conjugal transfer of *Agrobacterium tumefaciens* Ti plasmids (33, 63, 87) and extracellular virulence factors of *Pseudomonas aeruginosa* (34, 35). There is also evidence for homologous systems in *Erwinia carotovora*, *Rhizobium leguminosarum*, *E. coli*, and other gram-negative bacteria (see below). A considerably larger family of proteins exhibits homology with the carboxyl-terminal half of LuxR (Fig. 2). Although this larger group of proteins was originally described as the LuxR family (38), we propose that this group of proteins be designated the LuxR superfamily, and that only end-to-end LuxR homologs be included in the LuxR family.

The first hint that autoinduction is not restricted to luminous bacteria came in 1979 when it was reported that culture fluids from several nonluminous marine bacterial species contained a substance that mimicked the activity of HAI (36). However, at that time the structures of HAI and VAI had not been determined and the genes for luminescence had not been identified. Subsequent studies of autoinduction in *V. fischeri* have greatly facilitated recent studies of homologous cell density sensing systems in a number of other gram-negative bacteria. Because the *V. fischeri* system was the first to be reported and has been studied most extensively, it remains the

preferred model system for studies of the mechanism of autoinduction.

**Conjugal transfer of the *A. tumefaciens* Ti plasmid.** The plant pathogen *A. tumefaciens* incites crown gall tumors on many plant hosts by transferring oncogenic DNA fragments from the Ti plasmid to plant cell nuclei (86). Certain transferred genes direct the synthesis and secretion of opines, which are consumed by *A. tumefaciens* as nutrients. Ti plasmids also mediate their own conjugal transfer between agrobacteria. Ti plasmid conjugal transfer occurs only within crown gall tumors, or in the presence of exogenous opines (62), and conjugal transfer (*tra*) genes are coregulated with opine degradation genes (46). Conjugation also requires high donor cell densities and is controlled by a LuxR-LuxI type protein pair designated TraR and TraI (33, 63).

Transposon-generated mutants that are opine independent for conjugal transfer have been isolated in two different Ti plasmids, the so-called nopaline- and octopine-type plasmids (33, 63), and studies of these mutants revealed a regulator, TraR, which activates the expression of Ti plasmid *tra* genes (33, 63). The TraR and LuxR sequences show similarity over their entire lengths (Fig. 3A). Opine-induced cultures of *A. tumefaciens* release a diffusible compound that stimulates Ti plasmid transfer. This compound, *N*-3-(oxo-octanoyl)-L-homoserine lactone (Fig. 1), is similar to VAI, but with an eight-carbon acyl moiety (87). For consistency, we shall refer to this compound as the *Agrobacterium* autoinducer (AAI). TraR requires AAI to activate *tra* genes (33, 63). The Ti plasmid-encoded AAI signal generator TraI (33) is homologous to LuxI (Fig. 3B). Putative TraR-AAI complexes activate at least two *tra* operons and also activate the *traR* and *traI* genes themselves (33), thereby creating a positive-feedback loop analogous to that of *V. fischeri*. However, unlike the *V. fischeri luxR* and *luxI* genes, *traR* and *traI* are unlinked. Ti plasmid conjugation by opine-induced bacteria can be strongly stimulated either by high densities of donor bacteria or by exogenous AAI (33).

The *traR* gene of octopine-type Ti plasmids is positively regulated by the octopine-responsive transcriptional activator OccR (33), which also activates the octopine catabolism operon (83). A similar pattern of regulation is thought to occur in nopaline-type Ti plasmids. The conjugation-inducing opine in these plasmids is agrocinopine, and the agrocinopine catabolism repressor AccR (6) probably represses *traR* transcription in the absence of agrocinopine (32). This explains how opines control Ti plasmid conjugal transfer. The AccR repressor and the OccR activator are unrelated, indicating that the opine regulation of each *traR* gene evolved independently.

**Extracellular virulence factors in *P. aeruginosa*.** The opportunistic pathogen *P. aeruginosa* infects tissues at wound and burn sites, as well as causing chronic lung infections of cystic fibrosis patients and immunocompromised individuals. Infection involves a battery of virulence factors including the elastin-specific proteases LasB and LasA. A regulatory gene designated *lasR* was isolated by screening a *P. aeruginosa* gene library for the ability to restore normal LasB activity to a *P. aeruginosa* strain with a pleiotropic protease deficiency (34). LasR is homologous along its entire length with the LuxR and TraR proteins (Fig. 3A). LasR also regulates *lasA*, *aprA* (alkaline protease A), and *toxA* (exotoxin A) (35, 81).

A second gene (*lasI*), which encodes a polypeptide homologous with LuxI and TraI, was found immediately downstream of *lasR* (60). The *lasR* and *lasI* genes are arranged in tandem orientation on separate transcripts. LasI directs the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (*P. aeruginosa* autoinducer [PAI] [61] [Fig. 1]). PAI and VAI show virtually no cross-functionality (61). LasR activates transcription of *lasB*

**A**

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LuxR   MKNINADDTYRIINKIKACRSNM IN.QC SDMTKMVK EY  AIIYPHSMVKSD
TraR        MQHWLDK TDLAAIEGDEC ILKTG ADIADH G TG  LHI.....QHRH
SdiA   MQDKDFFSWRRTK LRFQRNETAE VY.HE ELQAQ  DY  CVRHPVPFTRPK
LasR        MALVDGF .ELERSSGKL VS.AI QKMASD  SKIL GLLPKDSQDYEN
RhiR   MKEESSAVSNLVFDF SESASAKSKD VL.LL GKISQY  SY AISGIPSPIERIDS

LuxR   ISILD PKK RQY DDAM IKY   DYSNSMHSPIN N.IFENNAVW KSPNVIKE K
TraR   ITAYT HRQ GST FDKK EAL   KRARSRKHIFT SGEHERPTLS DERAFY  S
SdiA   VAFYT PEA VSY QAXM LAI  LNPENFSQGHLM .....NDDLFSEAQPL  A
LasR   AFIVG PAA REH DRAG ARV  U SHCTQSVLPIF EPSIYQ...T KQHEFF E S
RhiR   YFVLG SVG FDR RENN VHA   HLSKTCDHAFV SEALRDQKLD SRRVM E

LuxR   TS IT FSF IHT NM ICM  AHSEKDNYIDSLFLHACMNIPLIVP.SLVDNYRKI
TraR   DF IRS IIT IKT N. FSM  ASDKPVIDLDREIDAVAAAATIGQIHARISF RT.
SdiA   AH RR VHSVFNA QT GF  SRCSRRE.IPILSDELGLKMQLLVRESLMAL RLN
LasR   AA VY L TM HG R. F GA  SVEAENRAEANRFMESVLPTLWMLKDYA... QSG
RhiR   EFK ID FSV HT A. FQSIV GAEKVELSTCDR......SALYLMAAYAHSL RAQ

LuxR   NIANNKSNND....LTK  C ACE SSV SK GC ERT FHLT AC  NTT
TraR   .....TPTAEDAACVDP ATY  IM   DE GVK YNS RVKLRER  DVR
SdiA   DEIVMTPEMN....FSK    S  SA   LSI ENT NFHQK NG INAP
LasR   AGLAFEHPVSKPVVLTS     V  SY SV CHC EAN NFHMG IR GVT
RhiR   IGNDASRKIQALPMITT   IT  A   AI T   A GR HRT QNVIL IQ  NVV

LuxR   N COSISK ILT A DCPYFKN
TraR   S AHLTAL IRRK
SdiA   N TQVACY AAT
LasR   S RVAAIK VNL  TL
RhiR   N PQMIAESFRLRT R
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**B**

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LuxI   FT  KKS FLAIPSEE KGILS  YC         VVEN  S E NSNAE I AC
TraI   VSP YQHQNSY  HR  AE   C AIEDG   F ELSPT A TF
LasI   I  GRR ..EFDKKL GE  K  AC  KG  SVI E I  ALSPY  I C
ExpI   MLE FDVSYTLLSEKK SEE FT KET ON X CI  F   DNAT  GV

LuxI   DT..EN       DY KSV  E GQQSA KDPMI   GKN..SSKINN
TraI   GG..GR M  APS T  EE   ATGSLSATTA  T  DTTLPTGRAGR
LasI   DTPEAQ F   T Y KM  GKEA CSPHI    .NS..GXKGSL
ExpI   G...DQ ICSS EK YN ITG  EQIDI .EGKY  S  DKARSKTILGN

LuxI   SASEI K E IYKHAVSQ ITEY   STAI RF  IKVPCH I DKEIHV DTKS
TraI   HLA T AGIIE MAN YDEI  DLRF RI   PNT L..EPVA NTVA
LasI   SDC  AR ARR LQND IQTL  TVGV XX A DVS PH..LK IERA
ExpI   PVS  L TV KSK YDGV  VSHPNLTI  XIS  ..EQGMSEKHERV

LuxI   MS INEQFKKAVLN
TraI   AGH ADRKSFERVCPPGYRSIIADDNGRPLRSAA
LasI   RIELNAQTQIALYG
ExpI   YL F ADNESQDVLVRRINHNQEFVESKLREWPLSFEPNTEPVG
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**C**

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luxI     CC   T  GG    CG  A    GG
luxD    AA   G  TC    TT  G    GG
lasB    CC   C CAG   ACT GG    GG
traI1   CG   C - G    C - G    CA
traI2   AG   C - G    T - G    CA
traA    TG   C - G    C - G    CA

Consensus   A N N T G N A X S A T N X T R C A S R T
```
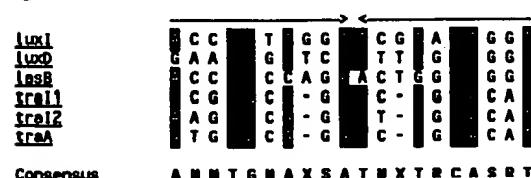
FIG. 3. Alignments of proteins homologous to LuxR (A), proteins homologous to LuxI (B), and of possible binding sites similar to LuxR-binding sites (C). Amino acid sequences were aligned using the PILEUP program of the Genetics Computer Group sequence analysis programs (17). Identical residues or conservative substitutions are indicated by black background. Inverted triangles indicate LuxR positions at which mutations affect interaction with VAI (75, 77). The stippled bar indicates a conserved HTH motif that may bind DNA. The amino acid sequences shown are LuxR from *V. fischeri* MJ1 (18, 31), TraR from an octopine-type Ti plasmid (33), SdiA from *E. coli* (84), LasR from *P. aeruginosa* (34), RhiR from *R. leguminosarum* biovar viciae (15), LuxI from *V. fischeri* (31), TraI from an octopine-type Ti plasmid (33), LasI from *P. aeruginosa* (60), and ExpI from *E. carotovora* subsp. *carotovora* (64). Possible binding sites are from *V. fischeri luxI* (18) and *luxD* (74), *A. tumefaciens* octopine-type Ti plasmids carrying *traI* and *traA* (33), and *P. aeruginosa lasB* (7).

in the presence of PAI. The expression of an extracellular protease specifically at high cell density is quite logical. At low cell densities, the enzyme would exist at low concentration as a result of diffusion, and proteolytic degradation would not be sufficient to benefit *P. aeruginosa*.

**Autoinduction in *E. carotovora*.** The macerating plant pathogen *E. carotovora* colonizes vascular tissues of susceptible plants and produces a variety of cell wall-degrading enzymes required for virulence (39). Enzyme activity is stimulated during the late stage of culture growth (85). A search for mutants that fail to produce these exoenzymes led to the identification of ExpI (42, 64), a protein homologous with LuxI, TraI, and LasI (Fig. 3B). A plasmid-borne copy of *luxI* partially suppressed an *expI* mutation, and conversely, a plasmid carrying the *lux* genes but with a nonfunctional *luxI* gene conferred luminescence to wild-type strains of *E. carotovora* but not to *expI* null mutants (64). Additionally, enzyme production in *expI* mutants was rescued when these mutants were plated adjacent to *E. carotovora* or adjacent to an *E. coli* strain expressing either *expI* or *luxI* (42, 64). Plants inoculated with normally avirulent *expI* mutants accompanied by high doses of synthetic VAI developed areas of limited tissue necrosis (42, 64). As might be predicted, an autoinducer identical to VAI is synthesized by *E. carotovora* (4). VAI also activates production of the antibiotic carbapenem (Cap) in *E. carotovora*, and a class of Cap⁻ mutants is suppressed by coculture with a wild-type strain or by the addition of exogenous VAI (4).

As yet, no LuxR homolog has been conclusively identified, but an open reading frame downstream from *expI* shares sequence similarity with LuxR (64) and is likely to encode at least one VAI-responsive regulator. Preliminary studies suggest this protein is required for exoenzyme production but not for carbapenem synthesis (42).

**Regulation of rhizosphere genes.** Strains of *R. leguminosarum* bv. viciae form symbiotic, nitrogen-fixing nodules on beans and peas. This bacterium synthesizes an abundant 24-kDa protein called RhiA (rhizosphere protein A) when grown on the exterior of nodules and in culture, but not when isolated from the interior of nodules (15). In the laboratory, RhiA is expressed in a growth-phase-dependent fashion. The *rhiA* gene lies in a three-gene operon (*rhiABC*), expression of which requires the linked *rhiR* (rhizosphere gene regulator) gene. RhiR shows significant similarity to members of the LuxR family of proteins (Fig. 3A), although neither a LuxI homolog nor a diffusible factor has been identified (15). The *rhiA* and *rhiR* genes are closely linked to a large cluster of *nod* genes and are subject to additional regulation by *nod* gene regulators. The regulatory protein NodD represses *rhiR* expression in the presence of a flavonoid *nod* gene inducer (15).

**SdiA, a cell division regulator in *E. coli*.** The earliest discovered LuxR homolog is an *E. coli* protein involved in cell division, designated SdiA (suppressor of division inhibition A, previously designated UvrC-28 kDa) (38). SdiA positively regulates the *ftsQAZ* operon, whose products are involved in cell septation (84). This operon is expressed from several promoters, and SdiA activates the dominant promoter responsible for expression of *ftsQAZ* during active cell growth (66). Neither a *luxI* homolog nor a cognate autoinducer has been reported. The regulation of *ftsZ* by a LuxR homolog suggests that cell division in *E. coli* may be influenced by cell density.

**Conservation of structure and function among LuxR-type regulators.** LuxR homologs have moderate overall similarities (18 to 25% identity [Fig. 3A]). Clusters of stronger similarity are found in two regions of these proteins: (i) a region that aligns with the putative VAI-binding region of LuxR (75, 77) and (ii) the HTH-containing DNA-binding region (Fig. 3A).

This HTH region includes a motif recently defined as a probe helix that is thought be involved in protein-DNA major groove interactions in a number of eukaryotic and prokaryotic transcription factors (79). As discussed above, this HTH region is homologous to analogous regions from other known DNA-binding proteins, such as the DNA-binding region of the FixJ subfamily of the two-component response regulator family (38).

LuxR is thought to bind to a 20-bp inverted repeat, the *lux* box, located between the *luxR* and *luxICDABEG* operons, and to a similar sequence found within *luxD* (18, 74). These sequences closely resemble an 18-bp sequence upstream of the *A. tumefaciens traA* gene and two virtually identical sequences upstream of *traI* (33) (Fig. 3C). Disruption of one of the *traI* elements substantially reduced *traI* activation by TraR (33). Both the *A. tumefaciens* and *V. fischeri* elements are located approximately 40 bp upstream of their transcriptional start sites. A similar inverted repeat is found upstream of the *lasB* gene of *P. aeruginosa* (Fig. 3C). Taken together, this information suggests that these LuxR-type proteins may have similar DNA-binding properties.

**Conservation in the signal generators.** The family of LuxI homologs has a stronger sequence conservation (28 to 35% identity) (Fig. 3B) than does the family of LuxR homologs. This similarity appears to cluster into two regions, although the boundaries of these clusters are not well defined and their roles are unclear. *V. fischeri* LuxI may utilize S-adenosylmethionine and 3-oxohexanoyl acyl carrier protein as substrates for VAI synthesis (28). In light of the similarity of autoinducer structures (Fig. 1), it seems probable that the general catalytic mechanism of autoinducer synthesis has been conserved.

The abilities to synthesize and secrete autoinducer-like molecules appear to be common features of many gram-negative bacteria (4). Cell-free culture fluids from a variety of gram-negative bacteria were analyzed for the presence of VAI-like compounds by measuring bioluminescence of *E. coli* containing the *V. fischeri lux* gene cluster with an inactivated *luxI* gene. Some culture fluids stimulated high levels of expression and a number of these culture fluids were subsequently shown to contain VAI (4). Some bacteria showed low but detectable levels of *lux* activation, suggesting that they may synthesize either low levels of VAI or higher levels of molecules which are only weakly recognized by LuxR.

**Autoinduction circuits as regulatory modules.** LuxR-type regulatory genes are themselves generally controlled by some exogenous environmental stimulus. For example, *luxR* has a cyclic AMP receptor protein-binding site and is activated by cyclic AMP (21–23, 31). The LuxR protein is also influenced by other stimuli such as heat shock (1, 19). The *traR* genes of both octopine- and nopaline-type Ti plasmids are regulated by particular opines and their cognate opine-responsive regulators (32, 33), while *rhiR* is repressed indirectly by NodD (15, 20). Another general feature of at least some of these systems is a positive autoregulatory loop, such that LuxR homologs often activate either their own gene or their cognate autoinducer synthase gene or both genes. LuxR activates *luxI* and under some conditions weakly activates *luxR* (18, 73, 74). Similarly, TraR activates both *traR* and *traI* (32, 33). Therefore, we can imagine two conditions that must be met for target genes to be induced: first, some external environmental signal other than an autoinducer must be perceived, and second, the bacteria must be at sufficiently high cell density for the autoinducer to accumulate to a threshold concentration. Positive autoregulation may create a transcriptional switch that can be changed rapidly from an off state to a relatively stable on state. In other words, a higher cell density may be required

to switch on an autoinducible gene than to maintain subsequent expression of that gene. Autoinduction systems may therefore serve as signal amplifiers within a single bacterium as well as signal disseminators between bacteria.

**Other diffusible signal molecules in bacteria.** The production of antibiotics in some *Streptomyces* spp. depends upon diffusible butyrolactones structurally similar to VAI (40). The most intensely studied of these is A factor from *Streptomyces griseus*, which regulates a number of secondary metabolic processes including streptomycin synthesis and sporulation (40). At a superficial level, butyrolactone-mediated regulation appears similar to autoinducer-dependent regulation, but the two systems are actually quite distinct. While LuxR-type proteins activate transcription, the A-factor receptor is a repressor that is neutralized by A factor. A-factor synthase is not homologous to LuxI (41), and similarly, at least one butyrolactone receptor is not homologous to LuxR (58).

A number of oligopeptide signals are important in bacterial sporulation. For example, one or more peptides released from cultures of *B. subtilis* stimulate both sporulation and competence (71). Both phenomena occur efficiently only when cells are cultured at high cell densities, while the addition of spent culture fluids enhances these processes at low cell densities. These peptides may activate a two-component kinase that initiates a signal cascade leading to sporulation and/or competence (71). Another example is the A signal of *Myxococcus xanthus*, which consists of single amino acids (or a mixture), that triggers fruiting body formation (44). Peptide pheromones also play an important role in regulating the conjugal transfer of plasmids between strains of *Enterobacter faecalis* (14).

## CONCLUSIONS AND FUTURE DIRECTIONS

The LuxR-LuxI family as yet has relatively few described members, but a recent survey suggests that the list will grow (4). Interestingly, there is some evidence for multiple autoinduction circuits in a single bacterial strain. For example, Kuo and Dunlap (48) have discovered a rather weak *luxI*-independent cell density control of *V. fischeri* luminescence that appears to result from the production of a compound related to VAI. Synthesis of this autoinducer is not genetically linked to *luxI* (48). Recent studies with *V. harveyi* also suggest that there may be overlapping and mechanistically distinct autoinduction circuits that regulate *lux* gene expression in this bacterium (5). This raises the possibility that a single strain of bacteria may use multiple autoinduction systems, each having a different autoinducer.

As described at the outset, certain bacterial behaviors can be performed efficiently only by a sufficiently large population of bacteria. We describe this minimum behavioral unit as a quorum of bacteria. LuxR-LuxI type systems provide an effective though not unique way for bacteria to take a census of their numbers. The LuxR and LuxI homologs so far discovered play roles in cell density-responsive regulation during interactions between bacteria and plant or animal hosts. It will be interesting to determine whether new examples will follow this trend.

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